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(54) Title: DEVICE FOR SITE DIRECTED NEOVASCULARIZATION AND METHOD FOR SAME

(57) Abstract

The invention includes a device and method. The device is a site directed neovascularization device. The device includes a biocompatible support. The device also includes a biological response modifier for inducing neovascularization. The biological response modifier is adsorbed to the biocompatible support. The method is for directing *in vivo* neovascularization. The method requires adsorbing a biological response modifier for inducing neovascularization onto a biocompatible support. The step of contacting a therapeutically effective amount of the adsorbed biological response modifier to at least one selected tissue then occurs. The method then involves directing neovascular cell growth at the contacted, selected tissue for a sufficient time to obtain a vascular structure. The method of this invention is useful for developing artificial organs and other tissues including nerves in an organism, and for sampling of cells and re-implantation after genetically altering the cells to produce a desired product.

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DEVICE FOR SITE DIRECTED NEOVASCULARIZATION AND METHOD FOR SAME

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to a device and method for directing the formation of new blood vessels and artificial organs. Specifically, the invention relates to a device and method for directing neovascularization with a biological response modifier adsorbed onto a support.

2. Description of the Background Art

Angiogenesis is the formation of blood vessels *in situ* and involves the orderly migration, proliferation, and differentiation of vascular cells and occurs during development. Angiogenesis is an infrequent event in the

adult and is associated in adults with wound and fracture repair. Exceptions to this are found in the female reproductive system where this process occurs in the follicle during development, in the corpus luteum during ovulation, and in the placenta during pregnancy. These specific periods of angiogenesis are relatively brief and highly regulated in contrast to the angiogenic events associated with tumor growth and diabetic retinopathy. The endothelial cell is considered to be the primary cellular target for angiogenesis. Research efforts have concentrated on the identity of polypeptide factors that control endothelial cell proliferation. The heparin-binding growth factor (HBGF) family of polypeptides has gained general acceptance as initiators of angiogenesis especially during development.

The gene family for producing the heparin-binding growth factor family of polypeptides includes HBGF-1 (acidic fibroblast growth factor), HBGF-2 (basic fibroblast growth factor), and three additional HBGF-like structures, hst/KS, int-2, and FGF-5, each of which is encoded by an oncogene. The prototype HBGF polypeptides are potent inducers of endothelial cell migration and/or proliferation <u>in vitro</u> and are known to modulate the expression of endothelial cell derived proteases. Further, HBGF-1 and HBGF-2 are tightly adsorbed to the extracellular matrix presumably by their avid affinity for the glycosaminoglycan heparin. The association between the HBGF prototypes and heparin protect these polypeptides from proteolytic modification. It has been suggested that the extracellular matrix can be the major source of HBGF-1 and HBGF-2 and activation can require hydrolytic extraction from sites of attachment for biological activity.

Hayek, et al (1987) reported the in vivo effect of fibroblast growth factor in rat kidney. (Biochem. Biophys. Res. Commun. 147:876-880.) The initiation of angiogenesis by the direct stimulation of endothelial cell proliferation is presumed to be a result of the Class I heparin-binding growth factor (HBGF-I) and the Class II heparin-binding growth factor (HBGF-II). These polypeptides are potent endothelial cell growth factors in vitro and angiogenesis signals in vivo. These polypeptides exert their biological response in vivo through high affinity cell surface receptors. The HBGF-I and HBGF-II share a structural similarity of 55 percent and both are synthesized as polypeptides lacking an apparent signal peptide sequence. Human cells which express the HBGF-I mRNA transcript do not secrete the polypeptide in vitro. Further, HBGF-II has been shown to be associated with the extracellular matrix and heparin protects HBGF-I from proteolytic modification by plasmin.

PCT International Publication Number WO 87/01728 discloses recombinant fibroblast growth factors. These growth factors are examples of biological response modifiers. This disclosure identifies the importance of the growth factors for constructing vascular systems in healing tissues. The invention of this disclosure is directed to recombinant DNA sequences for encoding bovine and human acidic and basic FGF and vectors bearing these DNA sequences. This publication does not disclose a device or method for site directed neovascularization.

The article, Van Brunt, et al., "Growth Factors Speed Wound Healing", Biotechnology 6 (1988):25-30, discloses the usefulness of growth factors in the

angiogenesis of damaged tissue. This article discloses a sponge implant model for wound healing in animals. The sponge consists of an inert polyvinyl alcohol that is implanted under the skin of the animal. Growth factor is then injected directly into the sponge. The wound undergoes rapid healing and an increase in blood vessels occurs at the wound site. The blood vessels resulting from this invention do not form complete, permanent vascular structures that are directed by a support to which the growth factor is adsorbed. This article does not disclose a device or method for site directed neovascularization.

U.S. Patent Number 4,699,141 to Lamberton, et al. discloses a container and method for neovascularization. This invention has a sponge body that is wetted throughout with a solution of fibrinogen and heparin. The sponge body is placed adjacent to or around a noncapillary blood vessel. Capillaries then grow into the sponge. The sponge can then be used as a receptacle for desired cells such as pancreas cells. This patent does not disclose a device or method wherein the growth of blood vessels is directed in a specific direction or between specific sites. Neither the heparin nor collagen in this invention modify a biological response. Both the heparin and collagen are substrates upon which a biological response modifier acts. The capillary growth developed by this invention is a result of the inflammatory response of the vessel to a foreign body or the sponge. The blood vessels of this invention are not directed in their growth and do not form permanent structures or long term structures. These blood vessels

are not permanent because the fibrinogen support is absorbed by the organism before maturation of the blood vessels can occur.

The blood vessels developed by the Lamberton, et al. invention are, essentially, a bundle of cells or capillaries within a sponge. This invention is identified as being a receptacle for "desired cells." Such a receptacle is desirable for developing an "artificial organ". The development of the receptacle requires an undesirably long period of time of about 6 weeks.

Genetically altered or unaltered cells provide a desired metabolic effect. Examples of gene transfer technology to produce altered cells are provided in the following three articles: Wolff, et al., "Expression of Retrovirally Transduced Genes in Primary Cultures of Adult Rat Hepatocytes", Proc. Natl. Acad. Sci. USA 84 (May 1987): 3344-3348; Ledley, et al., "Retroviral Gene Transfer into Primary Hepatocytes: Implications for Genetic Therapy of Liver-Specific Functions", Proc. Natl. Acad. Sci. USA 84 (1987) 5335-5339; and Wilson, et al., "Retrovirus-Mediated Transduction of Adult Hepatocytes", Proc. Natl. Acad. Sci. USA 85 (May 1988) 3014-3018. The art is lacking a satisfactory means to transfer genetically altered or unaltered cells into an organism and maintain those cells permanently within that organism such that the organism benefits from the desired metabolic effect of the cells.

The field of angiogenesis has been severly limited by the absence of devices and well defined methods for the selective demonstration of new blood vessel or "neovessel" growth. The importance of site-directing physiological neovessel formation has been long recognized in medicine. The prior art has indicated the possibility of such a process, but does not provide a neovessel design in the form of physiological embodiments for this purpose.

The invention is an in vivo site directed neovascularization device. The device includes a support. The support can be an absorbable support, a non-absorbable support, or both. The device also includes a biological response modifier for inducing neovascularization. The biological response modifier is adsorbed to support.

The invention also includes a method for directing in vivo neovascularization. The method requires adsorbing a biological response modifier for inducing neovascularization onto a support. The step of contacting a therapeutically effective amount of said adsorbed biological response modifier to at least one selected tissue then occurs. The method then involves directing or culturing neovascular cell growth at the contacted, selected tissue for a sufficient time to obtain a vascular structure.

The method of this invention is useful for providing artificial organs.

Objects of the present invention are to provide: (1) a new device for inducing site-directed neovascularization; (2) a method for in vivo formation of new blood vessel or a vascular bed; (3) mammalian cells collected about the implanted device of the present invention for multiplication, cloning, manipulation and implantation thereof; (4) a vascular bed for transplantation; and (5) other objects made evident from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates ECGF binding to collagen supports.

Figure 2 illustrates the effect of implanting ECGF immobilized on collagen sponges and the results thereof (arrows to sponges) are shown.

Figure 3 illustrates the H & E histological stain of sponges (IP in rat) are shown.

Figure 4 illustrates the site-directed gelfoam implant (Sg) with GF (growth factor) between liver (left, L) and spleen (right, Sp).

Figure 5 illustrates genetically engineered rat hepatocytes recovered from collagen sponges adsorbed with ECGF at 4 to 6 weeks of post-implantation.

Figure 6 illustrates a cross-section of a blood vessel developed according to this invention.

Figure 7 illustrates an angiogeneic response induced by HBGF-1 <u>in situ</u> four weeks after surgery.

Figure 8 illustrates the posterior portion of a fiber implant containing vascular strings that are generally connected to the mesentary tissue around the bowel loop.

Figure 9 illustrates multiple vascular connections between the fiber implant and mesenterial vessels and vascular turbosity within the implant.

Figure 10 illustrates an x-ray view of the multiple vascular connections of Figure 9.

Figure 11 illustrates a histological examination of a longitudinal section that reveals the presence of multiple vascular lumina surrounded by thick, collagenous and muscular walls of the neovessel structure.

Figure 12 illustrates the vascular bundle of Figure 6 at higher magnification which reveals the rich collagen component of the vascular structure and abundance of endothelial cell-lined capillary structures.

Figure 13 illustrates serum bilirubin levels of a Gunn rat implanted with hepatocytes seeded onto collagen (Type IV) and HBGF-1 coated PTFE fibers.

Figure 14A illustrates a Gortex shunt tube, containing a collagen I (Gelfoam) sponge, impregnated with HBGF-1, implanted onto the aorta of a rat for one month, then excised and cross-sectioned.

Figures 14B, 14C and 14D illustrates a Gortex shunt tube containing a bundle of Gortex angel-hair fibers coated with Type I collagen and impregnated with HBGF-1.

DETAILED DESCRIPTION OF THE INVENTION

The invention includes both a composition or "device" and a method for using that device. The device is used in vivo to stimulate and direct neovascularization. The neovascularization is accompanied by the growth of other cellular tissue including nerves. The device requires a support. The support must be capable of adsorbing a biological response modifier or adhering to a composition that can adsorb a biological response modifier. The biological response modifier is a compound that stimulates and induces neovascularization. The invention further includes a method for inducing neovascularization that can include the development of artificial organs and/or genetically engineered tissues.

A biological response modifier can be at least one compound or agent that stimulates or facilitates vascular cell growth from a tissue or organ. In other words, a biological response modifier is a biochemical agent, such as a growth factor, hormone, or their chimeric derivative, that directly or indirectly induces a transcriptional or translational cellular event. A biological response modifier directly or indirectly exerts an effect through a high affinity receptor. This effect produces vascular cell growth. Compounds that exert a direct stimulation of a receptor include hormones. Compounds that provide indirect stimulation of

a receptor include hormone prototypes or precursors and hydrolases. Hydrolases, such as a plasminogen activator, collagenase, or heparinase, initiate a biological response by enzymatically activating or releasing latent, stored, or zymogen precursors of direct biological response modifiers.

Biological response modifiers desirable angiogenic growth factors include a member of the group consisting of HBGF-I, HBGF-II, platelet-derived growth factor (PDGF), macrophage-derived growth factor (MDGF), epidermal growth factor (EGF), tumor angiogenesis factor (TAF), endothelial cell growth factor (ECGF), fibroblast growth factor (FGF), hypothalamus-derived growth factor (HDGF), retina-derived growth factor (RDGF), and mixtures thereof. The preferred embodiment of the invention uses HBGF-I. Desirable hydrolases include a member selected from the group consisting of heparinase, collagenase, plasmin, a plasminogen activator, thrombin, heparatinase, and mixtures thereof.

Hormones such as the growth factors are particularly desirable for use in this invention as biological response modifiers. Hormones specifically elicit cell growth and differentiation. The use of hormones as biological response modifiers cause neovascularization to rapidly occur and to form a complete vascular structure. The resulting blood vessel stimulated by hormones is more than just a mass of cells in that it has a tubular cavity and connective tissue between its cells. The resulting blood vessel produced

from the use of hormones is complete within itself and can be excised and transplanted into another portion of the body. The other biological response modifiers produce similar results, but do not provide as rapid a growth as hormones and, in particular, the HBGF-I and HBGF-II hormones.

The invention includes a biocompatible support to which the biological response modifier is adsorbed. The support can be either or both an absorbable or non-absorbable biocompatible matrix. The support must be implantable into an organism and is, desirably, rigid and strong enough to be transplantable after neovascularization has occurred. The biocompatible support must have the rigidity and strength to support neovascularization. Examples of absorbable supports include a member selected from the group consisting of collagen Type I, known commercially by the trade name "Gelfoam", laminins, fibronectins, gelatins, glycosaminoglycans, glycolipids, proteolipids, mucopolysaccharides, glycoproteins, polypeptides, and mixtures thereof. Examples of non-absorbable matrices include members of the group consisting of nylon, rayon, dacron, polypropylene, polyethylene, expanded PTFE, cross-linked collagen Type IV, and mixtures thereof. It is desirable that a selected support contain extracellular matrix protein to provide or to facilitate the adsorption of the biological response modifier to the biocompatible support.

An extracellular matrix protein can be the material from which the biocompatible support is formed or a component added to the biocompatible support to fully provide or, alternatively, facilitate the adsorption of the biological response modifier to the biocompatible support. An extracellular matrix protein component can include a pure or mixed composition of proteins or polypeptides. The proteins and polypeptides can be either natural or synthetic. The extracellular matrix protein component is desirably derived from extracellular structural molecules. These extracellular structural molecules include a member selected from the group consisting of collagens, laminins, fibronectins, gelatins, glycosaminoglycans, glycoproteins, proteoglycans, and mixtures thereof.

Expanded polytetrafluoroethylene (PTFE) has been found to be most suitable non-absorbable support for this invention. This support provides the following benefits. PTFE has a general lack of an inflammatory response which is the basis for the current acceptance of PTFE in the surgical community. PTFE can be coated conveniently with various components of the extra

cellular matrix which can adsorb a biological response modifier. Biologically active HBGF-1 and HBGF-2 can be immobilized to collagen-coated PTFE by previously established methods. PTFE polymers are routinely engineered to various specifications to meet a multitude of required configurations.

The configuration of the non-absorbable PFTE is a more critical aspect of the long-term implant model. All multicellular organisms utilize a three-dimensional architecture of branching fiber networks to solve the problem of increasing surface area in a given volume. Seeding of such a network with HBGF polypeptides before implantation allows for high localized concentrations of the mitogen. Non-woven multifilament angel-hair fibers of expanded PTFE are commercially available from W.L. Gore and Associates, Inc., Flagstaff, Arizona. These fibers allow sufficient organized surface area for infiltrating cells to be exposed to the environment of the host. This permits the free exchange of nutrients and toxic waste to occur while neovascularization processes occur. Furthermore, cell shape as determined by cytoskeletal components and attachment to a specific matrix generally is regarded to play a significant role in both cell proliferation and differentiation.

A support can be provided for use in this invention in any desired shape and size. A support as small as one $1mm^2$ is suitable to provide a base for neovascularization. Desirable shapes for a support can

be a strip, a sponge, or a tube. Supports are desirably capable of being secured within an organism. Suitable means for securing a support can include a staple, biocompatible glue, or other surgical procedures such as suturing or tying the support to a tissue.

A desirable support is obtained by filling a tube or sleeve of expanded PTFE with expanded PTFE fibers or "angel hair". Supports formed from tubes or sleeves provide a pouch for an artificial organ. The tubular form of the support and the bundle of fibers within the tube are particularly desirable for directing neovascularization. Such embodiments can be receptacles for implanted cells when the invention is used to provide an artificial organ.

The most effective concentrations for a biological response modifier can be any concentration that elicits a growth response from the target cells, but is not toxic to those cells. Effective or therapeutic concentrations of angiogenetic growth factors are between about 1 to about 10 nanograms per cubic millimeter of a support. A support for this calculation includes both the absorbable support and the non-absorbable support.

A support is provided in an amount suitable to establish the length and width of the desired blood vessel. For example, if a blood vessel is desired between two tissues and there exists a distance between those two tissues, then a corresponding length of support is

implanted into the organism to provide the approximate length and width of this desired blood vessel. The amount of the biological response modifier is then adapted to the amount of support required to form this basic structure.

The invention can be practiced without a non-absorbable support. For example, a complex with gelatin, HBGF-1, or HBGF-2 is capable of inducing neovascularization in vivo at polypeptide concentrations consistent with the demonstration of this biological activity in vivo. This neovascular response is capable of sustaining induced site-specific neovessel formation for up to four weeks in the neck and peritoneal cavity of the rat. However, the device of this invention without a support has limited utility for the induction of long-term neovessels. This is because the three-dimensional architecture of the collagen sponges is ultimately disrupted by a reabsorption process that occurs within three to four weeks after implantation. Nonabsorbable solid polymeric supports of well-defined specifications and containing bonded components of extracellular matrices induced the expression of long-term stable neovessels in vivo. An example of such an embodiment is a nonabsorbable support bonded with both collagens Type I and Type IV and having both HBGF-1 and HBGF-2 attached to the collagens.

A neovascularization device can also be seeded with desired cells prior to or subsequent to implantation in a host. In a preferred embodiment, such cells are mammalian cells and express a protein capable of performing a particular function. The cells can be genetically engineered cells capable of expressing a heterologous protein. Alternatively, the cells can be naturally occurring cells capable of providing a desired function or functions such as hepatocytes.

Desirable embodiments of the invention have cells seeded in or on the neovascularization device which are genetically engineered to express at least one heterologous protein. Such a protein is preferably a therapeutic agent. The expressed protein may or may not be secreted from the genetically engineered cells.

The genetically engineered cells used with this invention are transformed with at least one gene that encodes for the desired heterologous protein. The cells are transformed with a suitable vector or expression vehicle which includes the desired gene. The vector can also include a promoter for expression in the host cells. In mammalian cells, the promotor for expression can be SV 40, LTR, metallothionein, PGK, CMV, ADA, TK, or others. The vector can also include a suitable signal sequence or sequences for secreting the therapeutic agent from the cells. The selection of a suitable promotor is deemed to be within the skill of the art.

The vector or expression vehicle is preferably a viral vector and in particular a retroviral vector. Representative examples of suitable viral vectors, which can be modified to include a gene for a therapeutic agent, include Harvey Sarcoma virus, ROUS Sarcoma virus, MPSV, Moloney murine leukemia virus, DNA viruses such as adenovirus and others. Alternatively, the expression vehicle can be a plasmid. Transformation can be accomplished by liposome fusion, calcium phosphate or dextran sulfate transfection, electroporation, lipofection, tungsten particles, or other procedures. The selection of a suitable vehicle for transformation is deemed to be within the scope of those skilled in the art.

When a retroviral vector is employed as the expression vehicle for transforming cells, steps should be taken to eliminate and/or minimize the chances for replication of the virus. Various procedures are known in the art for providing helper cells which produce viral vector particles that are essentially free of replicating virus. Examples of such procedures are found in Markowitz, et al., "A Safe Packaging Line for Gene Transfer; Separating Viral Genes on Two Different Plasmids", Journal of Virology 62(4) (April 1988):1120-1124; Watanabe, et al., "Construction of a Helper Cell Line for Avian Reticuloendotheliosis Virus Cloning Vectors", Molecular and Cellular Biology 3(12) (Dec. 1983):2241-2249; Danos, et al., "Safe and Efficient Generation of Recombinant Retroviruses with Amphotropic

and Ecotropic Host Range", Proc. Natl. Acad. Sci. 85 (Sept. 1988):6460-6464; and Bosselman, et al., "Replication-Defective Chimeric Helper Proviruses and Factors Affecting Generation of Competent Virus; Expression of Moloney Murine Leukemia Virus Structural Genes via the Metallothionein Promoter", Molecular and Cellular Biology (5) (May 1987):1797-1806 disclose procedures for producing a helper cell which minimizes the chances for producing a viral particle that includes replicating virus. This procedure and other procedures can be employed for genetically engineering cells by use of a retroviral vector. In addition to the promotor and the gene for the therapeutic agent, other material can be included in the vector. This material can include a selection gene such as a neomycin resistance gene, a sequence for enhancing expression, or other materials.

Genetically engineered mammalian cells can be implanted in a mammal by use of a neovascularization device. These genetically engineered cells are desirably implanted into a mammal of the same species. In a preferred embodiment, the genetically engineered mammalian cells are cells originally derived from a patient, genetically engineered to include a gene for at least one therapeutic agent, and implanted into the patient from which they were derived by use of a neovascularization device in accordance with the invention. These autologous genetically engineered cells then provide "gene therapy" by *in vivo* production of the therapeutic agent for treatment of the patient.

The genetically engineered cells can be engineered such that the therapeutic agent is secreted from the cells in order to exert its effect upon cells and tissues either in the immediate vicinity or in more distal locations. Alternatively, the therapeutic agent, if it is not secreted from the engineered cells, exerts its effect within or on the engineered cells and can cause the metabolism of substances that diffuse into or onto the cells. Examples of such therapeutic agents include adenosine deaminase (ADA) that functions within the cell to inactivate adenosine, a toxic metabolite that accumulates in severe combined immunodeficiency syndrome, or phenylalanine hydroxylase that functions within a cell to inactivate phenylalanine, a toxic metabolite in phenylketonuria.

The genetically engineered cells used with this invention are transformed with a gene for at least one heterologous protein. This protein is preferably a therapeutic agent. The term "therapeutic agent" is used in its broadest sense and means any agent or material which has a desired or beneficial effect on the host. The therapeutic agent can be more than one type of protein. Desirable proteins include CD-4, Factor VIII, Factor IX, von Willebrand Factor, TPA, urokinase, hirudin, the interferons, tumor necrosis factor, the interleukins, hemotopoietic growth factors including G-CSF, GM-CSF, IL3, erythropoietin, antibodies,

glucocerebrosidase, ADA, phenylalanine hydroxylase, human growth hormone, insulin and others. The selection of a suitable gene is deemed to be within the scope of those skilled in the art. Mixtures of cell types can also be used with this invention such s genetically engineered smooth muscle cells, fibroblasts, glial cells, keratinocytes, or others.

The effect in genetically engineered cells when used in gene therapy, can be controlled by the selection of high producing clonal populations and/or the use of vectors with enhanced expression. This can provide, in vivo, therapeutically effective amounts of a desired therapeutic agent for treating a patient. In determining the number of cells to be implanted, factors such as the half life of the therapeutic agent, volume of the vascular system, production rate of the therapeutic agent by cells, and the desired dosage level are considered. The selection of such vectors and cells is dependent on the therapeutic agent and is within the scope of those skilled in the art.

The neovascularization device of the invention can also be employed to obtain cells from a host by implanting the device in a host and after a period of time removing the implanted neovascularization device from the host for recovery of cells which have been collected on the device. Such cells can be differentiated and used for a variety of purposes. For

example, this procedure can provide a source of autologous cells for genetic engineering and subsequent return to the host as genetically engineered cells for expression of a protein. Cells collected in this manner can be genetically engineered and then returned to the host to provide an artificial organ.

The process for directing neovascularization first involves preparing the device of this invention as described above. The device is prepared by adsorbing a biological response modifier, that is suitable for inducing neovascularization, onto a support. The biological response modifier must be present on the support in such a concentration as to be therapeutically effective for eliciting cell growth. The adsorbed biological response modifier is then contacted to at least one selected tissue. Typically, the device is connected to at least two separate sites between which a blood vessel is desired. These two sites can be the same or separate tissues or organs. The method then involves culturing neovascular cell growth at or from the contacted tissue. Culturing of the contacted cells must occur for a sufficient time to allow or enable neovascularization and the vascular structure to form.

Figure 1 demonstrates that ECGF binds to collagen supports. This is shown by an elution profile of HBGF-1 (ECGF) from collagen type IV-Sepharose and gelatin-Sepharose columns. Collagen Type IV-Sepharose and The gelatin-Sepharose (1 ml) were packed in a column

and washed with 5 mls of 2M NaCl in 50mM Tris HCl, pH 7.4, followed by an exhaustive wash with 50mM Tris HCl, pH 7.4 (adsorbtion buffer; AB). The Gelatin-Sepharose was from Pharmacia. Bovine collagen-Type IV-Sepharose was obtained from Sigma Chemical Company, St. Louis, MO. and (^{125}I)-HBGF-1 was prepared as previously described. (^{125}I)-HBGF-1 (approximately 5X10^{5} cpm) in absorption buffer was added to the column in a volume of approximately 0.1 ml and the column washed with absorption buffer. Elution of column-associated (^{125}I)-HBGF-I was achieved with 1.5M NaCl in absorption buffer or 50 units of heparin (Upjohn, Kalamazoo, MI) in absorbtion buffer. The NaCl-eluted column was regenerated with an absorption buffer wash and the heparin-eluted column was regenerated by consecutive washes with 1.5M NaCl in absorption buffer followed by another wash with absorbtion buffer. The matrix affinity procedures were performed at room temperature (about 22°C to 25°C).

Figure 2 demonstrates that ECGF binds to collagen supports. The adsorbed factor was implanted in various anatomical sites to demonstrate the practicality of using growth factor-adsorbed implants to stimulate neovessel formation and the growth of vascular beds in areas of interest. The effect of implanting ECGF immobilized on collagen sponges and the results thereof (arrows to sponges) are shown:

A. Neck, 2 weeks, no ECGF;

B. Neck, 2 weeks, plus ECGF;

C. IP, 2 weeks, no ECGF;

D. IP, 2 weeks, plus ECGF;

E. IP, 2 weeks, plus ECGF site-directed; and

F. IP, 2 weeks, plus ECGF implantation in omentum.

Figure 3 demonstrates that the device of this invention induces significant angiogenesis in situ. These implants were removed at various times for examination by common methods of histology in order to determine the microscopic nature of these dynamics. The following abbreviations are used: Sg represents "sponge (C-1)"; Sp represents "spleen"; L represents "liver"; and BV represents "blood vessel (aorta)". H & E histological stain of sponges (IP in rat) are shown:

A. sponge--two weeks, IP, without ECGF;

B. sponge--one week, IP, plus ECGF;

C. sponge--two weeks, IP, plus ECGF;

D. sponge glued to liver, 2 weeks, plus ECGF;

E. sponge glued to spleen, 2 weeks, plus ECGF; and

F. sponge wrapped around aorta, 2 weeks, plus ECGF.

Figure 4 demonstrates that ECGF induces significant and stable angiogenic response in situ by the recruitment of appropriate cell types as shown in Figures 2 and 3. Implants were established to create site-directed bridges between a large variety of organs, vessels, tissues and the like. Illustrated are the site-directed Gelfoam implant (Sg) with growth factor (GF) between liver (left, L) and spleen (right, Sp).

Figure 5 demonstrates that the device of this invention serves to create neovessels independent of the implantation site in situ. The device has an ability to serve as a recruitment vehicle for mammalian cells in general and as a vehicle to maintain the viability and physiological environment for and of the implanted and transplanted cells. Genetically engineered rat hepatocytes recovered from collagen sponges adsorbed with ECGF after 4 to 6 weeks post-implantation are shown. Hepatocytes were removed to determine their viability.

Figure 5A shows the results with no growth factor. Note that in Figure 5A few cells appear to be unhealthy and there is no proliferation or growth of survivor cells. Figure 5B shows the results with growth factor. Note that in Figure 5B healthy viable cells are accompanied by significant proliferation.

The device and method of this invention can provide angiogenesis and neovascularization from one or more sites on a single tissue or different tissues. The development of a blood vessel from a single site of one

tissue, such as an artery, provides a vessel that can be transplanted or that can be used as an artificial organ. The development of a blood vessel between two or more sites located on the same or different tissues provides improved circulation between the sites.

Figure 6 illustrates a cross section of a blood vascular structure developed by the device and method of this invention. This figure demonstrates that the blood vessels developed by this invention are not merely a bundle of vascular cells growing in an undirected manner. The blood vessel 1 contains endothelial cells 2, mesothelial cells 3, pericytes 4, smooth muscle cells 5, fibroblasts 6, and neuronal-like cells 7. The cross section of the blood vessel 1 demonstrates the formation of capillary-like structures 8, arteries 9, and vein-like structures 10. This development of a complete vascular structure provides a rigid vessel that remains permanently in the organism and that can be transplanted within this organism.

A method of this invention can be used to provide an artificial organ by first directing the growth and development of a blood vessel from a tissue. The developed blood vessel is then injected or seeded with cells from a selected tissue or organ. The injected cells can be genetically altered before being seeded into the blood vessel. The seeded cells can provide a desired metabolic effect. These metabolic effects can include

hepatic functions such as bilirubin metabolism and pancreatic functions such as insulin production. Other metabolic functions can be provided by cells containing one or more hormone producing genes. Artificial organs developed according to this invention can provide desired functions without being subject to a response from the organism's immune system.

EXAMPLE 1

Example 1 demonstrates various embodiments of the device or composition of the invention and the method by which the device is produced. This example uses HBGF-I with a radioactive iodine marker. In therapeutic use, the radioactive marker would not be present. Example 1 is as follows.

Gelatin-Sepharose and collagen Type IV-Sepharose were examined for the ability to absorb (^{125}I)-HBGF-1. Figures 1C and G show that the majority or approximately 80 percent of the (^{125}I)-HBGF-1 binds to immobilized gelatin and collagen Type IV and can be eluted with 1.5M NaCl. Adsorbed (^{125}I)-HBGF-1 can also be eluted with 0.5M NaCl (data not shown). Denaturation of (^{125}I)-HBGF-1 by heating at 90^{o}C for 1 minute significantly reduces the ability of the polypeptide to bind to immobilized gelatin and collagen Type IV by inactivation of the binding domain within the HBGF-1 polypeptide structure.

The (^{125}I)-HBGF-1 adsorbed to immobilized gelatin and collagen Type IV can also be eluted with heparin as shown in Figures 1A and E. Approximately 20% of the growth factor, which remains bound after heparin elution, can be eluted with 1.5M NaCl.

Pretreatment of the gelatin and collagen Type IV matrix with 50 units of heparin significantly reduces the ability of either matrix to absorb (^{125}I)-HBGF-1 as shown in Figures 1B and F. Regeneration of either matrix with a 1.5M NaCl wash permits (^{125}I)-HBGF-1 adsorption.

Bovine serum albumin at 1mg per ml and human fibronectin at 1mg per ml do not significantly elute (^{125}I)-HBGF-1 absorbed to either matrix as shown in Figures 1D and H.

EXAMPLE 2

Example 2 demonstrates the method for implantation of the device of this invention and for eliciting neovascularization. The use of immobilized gelatin with HBGF-I represents the preferred embodiment of the invented method. Example 2 is as follows.

Example 2 demonstrates that HBGF-I binds to both immobilized gelatin and to collagen Type IV. It is shown that HBGF-I, adsorbed to gelatin sponges, promotes

angiogenesis in the rat at concentrations of the growth factor which are consistent with the growth factor's activity as an endothelial cell mitogen in vitro. This concentration is about 10^{-3} times lower than the concentration used in vitro in the art.

The abdomen of an anesthesized male rats weighing 250 grams was washed with 20 percent volume to weight (v/w) ethanol and an incision was made into the abdominal cavity wall to expose the abdominal cavity. Gelfoam, manufactured by Upjohn, Kalamazoo, Michigan, was cut into strips of approximately 5 by 20mm. The sponge was cemented to the distal area of the abdominal aorta with n-butylcyanoacrylate. A bridge was created with the free end of the sponge when the free end was cemented to another tissue. In the studies that were conducted to provide these examples, the following tissues were actually contacted by the device. These tissues were other organs including the liver, kidney, and spleen, the abdominal cavity, and other macro and micro vessels. Various concentrations of HBGF-1 from about 1 to about 10 ng per mm^3 were adsorbed to sponges for these studies. The surgical opening was closed with a staple gun. The animals were fed a normal diet and the incision was opened 1 week after surgery. The collagen sponge was surgically extracted, grossly examined for blood vessel formation and the sponge prepared for histological examination.

It is known that HBGF-1 binds to immobilized gelatin and collagen Type IV, therefore, the possibility was evaluated that commercial gelatin sponges sold by the tradename "Gelfoam" adsorbed with HBGF-1 could be utilized as a method for inducing angiogensis _in situ_. Survival surgery was performed on the rat in order to implant gelatin sponges which were treated with HBGF-1. HBGF-1-adsorbed Gelfoam was independently placed in the neck and peritoneal cavities in the rat. A significant angiogenic response was observed _in situ_ one week after surgery with 1ng HBGF-1 per mm^2 (Figure 2). Blood vessels, which migrated away from the tissue site of implantation, were observed macroscopically to be exclusively within the gelatin sponge. Control sponges without HBGF-1 and sponges adsorbed with HBGF-1 and heparin did not induce neovascularization after one week _in vivo_. The latter is consistent with the ability of heparin to prevent HBGF-1 adsorption to immobilized gelatin and collagen-Type IV. A titration curve with various concentrations of HBGF-1 was performed using this procedure and results similar to Figure 1 was observed with 1 to 10ng HBGF-1 per mm^3 of sponge (data not shown). Histological examination (Figure 3) of the sponge removed after one week _in situ_ revealed new blood vessel growth within the sponge.

Since HBGF-1-adsorbed Gelfoam alone (without more) is an efficient inducer of angiogenesis from the serosa. The ability of immobilized HBGF-1-adsorbed implants to induce and sustain the process of neovascularization within the peritoneal cavity was assessed. Separate

surgical implants were cemented as strips of Gelfoam to the abdominal aorta in the rat creating a bridge between this site and either the kidney, spleen, liver, or abdominal wall (Figure 4). After two weeks in vivo, the implants were examined for the extent of angiogenesis. Bidirectional formation of new blood vessels along the HBGF-1-adsorbed gelatin sponge from the liver and aorta was observed. Similar bidirectional results were observed with implants cemented from the aorta to either the kidney, spleen, or abdominal wall (data not shown). Histological examination of these implants yielded results identical to those observed in Figure 3.

Induced neovascularization within the peritoneal cavity was also shown to sustain the proliferative potential of a genetically engineered rat hepatocyte cell strain simultaneously implanted with the HBGF-1-adsorbed Gelfoam (Figure 5). Hepatocytes were grown to high density (10^8 cells) on a Gelfoam sponge. Prior to surgical implantation, 10ng of HBGF-1 per mm^3 of sponge was added. Control sponges did not contain any adsorbed HBGF-1. Separate surgical implants were cemented as a bridge between the liver and the spleen and allowed to remain in situ for four to six weeks. At this time, the implants were removed, digested with either trypsin or collagenase to recover implanted cells which were maintained in tissue culture. Cells which were recovered from HBGF-1-adsorbed Gelfoam sponges were able to proliferate in vitro under selective pressure which

reflected genetic disposition (Figure 5B). In contrast, the cells recovered from control Gelfoam sponges displayed a loss of proliferative potential (Figure 5A). Histological examination of sponges containing the cells revealed that HBGF-1 also induced a response similar to Figures 3 and 4.

In accordance with the device and method of the present invention, angiogenesis and neovascularization has been achieved between various tissues and organs as demonstrated by Figures 2 through 5. Neovascularization has been similarly accomplished between the following loci (data not shown): liver to spleen; liver to kidney; spleen to kidney; liver to aorta; liver to vena cava; liver to omentum (omentum, containing pancreatic tissue); aorta/to vena cava; spleen to aorta; spleen to vena cava; spleen to omentum kidney to aorta; kidney to vena cava; kidney to omentum; omentum to aorta; and omentum to vena cava.

EXAMPLE 3 AND COMPARATIVE EXAMPLE A

Example 3 demonstrates the device of the invention having a non-absorbable support. The experiments performed to derive this example were conducted with either Type I or Type IV collagen and involved implantation onto the liver or the spleen of a rat.

Comparative Example A demonstrates that the use of the same materials and procedures of Example 3 without HBGF-1 did not induce neovascularization.

HBGF-1 adsorbed, collagen-coated (Type I or IV) expanded PTFE fibers were surgically implanted in the peritoneal cavity (onto the liver or the spleen) of the rat. A significant angiogenic response was specifically induced by HBGF-1 in situ and the results four weeks after surgery are shown in Figure 7. Blood vessels, which have migrated from the tissue site of implantation, could be observed macroscopically within and around the implanted fibers. The anterior portion of the fiber implant, which was attached to the liver, exhibited substantial neovessel growth from the liver into the interior of the implant (Figure 7). Further examination revealed that the posterior portion of the fiber implant (attached to a specific organ) or regions in the vicinity of the implant contained vascular "strings" which were generally connected to the mesentary tissue around the bowel loop (Figure 8). It was also possible to induce and sustain long-term bi-directional neovessel formation between the liver and spleen by the implantation of separate HBGF-1-treated fibers on each organ. The ability of HBGF-1 adsorbed implants to maintain the neovessel structures within the peritoneum is evidenced by these highly vascular bridges. Control fibers of Comparative Example A did not induce neovascularization even after six months following surgical implantation. Titrations with various concentrations of HBGF-1 were performed using this procedure. Similar results were

obtained with HBGF-1 at concentrations between 1 to 100 ng/mm^3 of fiber surface area. The concentration of HBGF-1 required to induce an angiogenic response in the fiber implant model is consistent with the results obtained with the Gelfoam implant model and the mitogenic activity of the polypeptide *in vitro*.

EXAMPLE 4

Example 4 demonstrates that the blood vessel produced in Example 3 displayed a large organized solid matrix including a network of neovessel formations.

Two months following surgical placement of the HBGF-1-treated implant on the spleen of a rat, the abdominal organs were perfused and fixed (formaline) using a catheter placed in the lower thoracic aorta. Subsequently, the abdominal organs were perfused with a radio-opaque silicone rubber dye sold by the trademark, Microfil, followed by soft X-ray analysis (magnification 27KV). Multiple vascular connections between the fiber implant and mesenterial vessels were observed as well as a vascular turbosity within the implant which is typical for new vessel formation (Figure 9). Histological examination of the implant itself displayed a large organized solid matrix containing a network of neovessel formations interdigitated with different cell types, which is consistent with results previously obtained with the short-term HBGF-1-treated Gelfoam implant model. X-ray analysis of the long-term fiber implant as shown in Figure 10 has confirmed that neovessel formation within the fiber network has become integrated with the vascular tree of the host, primarily through the bridges

("strings") of richly vascular tissue (Figures 7 and 8). Histological examination of the longitudinal section through a typical vascular connection revealed the presence of multiple vascular lumina surrounded by thick, collagenous and muscular walls of the neovessel structure (Figure 11). Cross-sectional analysis through these vascular connections further related the presence of a monolayer of mesothelial cells surrounding a large vascular lumina in the central portion, encompassed by prominent endothelial cells and multiple layers of smooth muscle cells, representing mature and highly differentiated arteries. Venous lumina are less visible and present as partially collapsed slits. Within the periphery are abundant capillary lumina, and the entire vascular bundle is surrounded by a continuous fibrocellular capsule (Figure 6). Further examination of this resource at higher magnification revealed the relatively rich collagen component of vascular structure as well as the abundance of endothelial cell-lined capillary structures (Figure 12). The presence of two distinct, yet prominent, round structures, marked with asteriks were also observed. These structures displayed histological characteristics of neuronal-like structures. Collectively these data suggest that HBGF-1 is capable of signaling a variety of the squamous mesothelial cells of the serosa and the proximal cells of the tunica adventita to initiate angiogenesis. The appearance of mesoderm- and neuroectoderm-derived cells is consistent with the ability of HBGF-1 to act as a mitogen <u>in vitro</u> for epithelial cells, fibroblasts, smooth muscle cells, mesothelial cells, endothelial

cells, astrocytes and oligodendrocytes. The presence of neuronal-like structures is also consistent with the nerve growth factor (NGF)-like biological activity of HBGF-1 to induce neurite extension and survival of PC12 cells *in vitro*.

EXAMPLE 5 AND COMPARATIVE EXAMPLE B

Example 5 demonstrates that the presence of a large organized solid matrix, containing a network of mature muscular neovessel formations of Example 4 and which are contiguous with the host's vascular tree *in situ*, permits successful selective cell transplantation.

Comparative Example B demonstrates that the use of the same materials and procedures of Example 5 without HBGF-1 did not sustain selective cell transplantation.

Homozygous Gunn rats lack UDP-glucuronosyltransferase for bilirubin and cannot efficiently excrete bilirubin. For this reason, Gunn rats exhibit lifelong nonhemolytic unconjugated hyperbilirubinemia. In order to examine the genetic therapy potential of this system, hepatocytes were harvested by collagenase perfusion of syngeneic Wistar (RHA) rats. The Wistar rat is genetically identical to the Gunn rat except that it contains a normal bilirubin conjugation locus.

In Example 5, HBGF-1 adsorbed collagen (Type IV) coated PTFE fibers were implanted next to the liver and after ten to fourteen days the peritoneal cavity was

surgically opened revealing numerous neovessel formations both protruding from the liver and extending into the bundle of fibers (Figure 7) and connecting the bowl loop with richly vascular bridges. Primary hepatocytes harvested from syngeneic Wistar (RHA) rats were injected into the fiber network of the vascularized fibers. Immediately, serum bilirubin levels began to decrease and ten days after hepatocyte injections, the serum bilirubin levels had decreased by 50 percent. A gradual decrease to greater than 60 percent was observed for the duration of the experiment (60 days) as shown in Figure 13A. Experiments have determined that reduced levels of serum bilirurin (>60%) can be maintained at least 181 days and histological examination of these long-term implants contain viable hepatocytes. These data suggest that HBGF-1 fiber implant model functions _in vivo_ as a receptacle for the successful site-specific introduction of cells capable of expressing a differentiated physiologic function.

In Comparative Example B, the hepatocytes were seeded onto collagen (Type IV) coated PTFE fibers, which did not contain adsorbed HBGF-1, and surgically implanted on the right lobe of the liver. The serum bilirubin levels decreased to approximately 50 percent. This was followed immediately by a sharp reversion to the original serum bilirulrin level. Figure 13B shows that the serum bilirubin levels remained constant for the duration of the experiment (60 days). Histological examination of these implants after twenty days suggested that accumulating levels of toxic-like acids within the fiber implant led to the ultimate death of the transplanted hepatocytes.

The long-term HBGF-1 fiber implant model of Example 5 induces a prominent angiotropic and neurotropic response when appropriately implanted in the rat. Example 5 demonstrates the ability of HBGF-1 to induce, sustain, and maintain the anatomical coordination of highly sophisticated and widely diversified mammalian cell types *in vivo*. The interrelationships between extracellular matrix components and differentiation-specific gene regulation can provide information critical for genetic engineering therapies. This invention may also prove useful as a site-specific transgenic alternative with the ability to understand the temporal and coordinated expression of growth and differentiation signals during neuronal and angiogenic development in the adult.

EXAMPLE 6

Example 6 demonstrates the neovascular device of this invention wherein genetically engineered cells are seeded into the device. Example 6 is as follows.

A. The construction of the pG2N retroviral vector, that was used to genetically engineer endothelial cells to produce rat growth hormone, was performed with SV40 promoted neomycin resistance gene and a rat growth hormone cDNA. These were placed into the pB2 retroviral vector provided by the Laboratory of Molecular Hematology at NIH. A growth hormone cDNA was obtained by digesting the plasmid RGH-1 according to *Nature* 270 (1977):494 with

Xho I and Mae II restriction endonucleases from Boehringer Mannheim Biochemicals. This rat growth hormone cDNA was electrophoretically isolated out of an agarose gel and purified via binding/elution to glass beads sold by the tradename, Geneclean Bio, 101, La Jolla, California. This growth hormone cDNA was then blunted using the large fragment of DNA polymerase Klenow known by the name, from New England Biolabs and nucleotide triphosphates as recommended by the manufacturer. This fragment was then purified with Geneclean product.

The B2 vector was constructed in order to replace the Neo^R gene in N2 according to M.A. Eglitis, et al., Science 230 (1985):1395; D. Armentano, et al., J. Virol 61 (1987):1647 with a multiple cloning site. N2 was first digested with Eco RI, thereby releasing both the 5' and 3' LTRs with the adjoining MoMLV flanking sequences. The 3' LTR fragment was ligated into the EcoRI site of the plasmid GEM4 from Promega Biotech. The 5' LTR fragment with its flanking gag sequence was then digested with Cla I, Hind III linkers were added, and the fragment was inserted into the Hind III site of pGEM4.

The pB2 vector was digested with the HincII restriction endonuclease from New England Liolabs, and phosphatased using calf alkaline phosphatase from Boehringer Mannheim Biochemicals. The pB2 plasmid was then purified with the Geneclean product. The pB2 vector and the rat growth hormone cDNA were then ligated using T4 ligase from New England Biolabs, pG2 was then digested

with BamHI from New England Biolabs, purified with the Geneclean Bio 101 product, and blunt ended with the Klenow fragment. A 340 base pair SV40 promoted neomycin resistance gene fragment was isolated from the pSV2CAT plasmid (ATCC accession number 37155) by digesting with PvuII and HindIII from New England Biolabs. This fragment was isolated by agarose gel electrophoresis and purified with the Geneclean product. The SV40-neomycin resistance fragment was then ligated using T4 ligase from New England Biolabs with pG2 and transformed into DH5 competent bacteria per the manufacturer's instructions (BRL). Colonies were screened and the resulting plasmid construct was called pG2N. The SAX vector was obtained as described in Proc. Natl. Acad. Sci. USA 83 (1988):6563.

The recombinant vectors, N2, SAX, G2N, used in this example were each separately transfected into the currently available retroviral vector packaging cell lines, including the amphotropic packaging lines, PA317 Mol. Cell. Biol. 6(1986):2895, and the ecotropic line, Psi2, Cell 33(1983):153. These lines were developed in order to allow the production of helper virus-free retroviral vector particles.

B. The CD4 containing plasmid, p4B, which was a gift of Richard Axel of College of Physicians and Surgeons Columbia University, New York, New York, was digested with the restriction endonucleases Eco RI and Bam HI from New England Biolabs, Beverly, Massachusetts, to release the CD4 gene which was isolated by agarose gel

electrophoresis followed by purification via binding/elution to glass beads using the Geneclean product, Bio 101, La Jolla, California, in the manner recommended by the manufacturer. The CD4 fragment was ligated, using T4 DNA ligase as recommended by the supplier, into Eco RI plus Bam HI cut Bluescript cloning vector from Stratagene Co., La Jolla, California. The ligation was then transformed into competent DH5 alpha bacteria from Bethesda Research Labs, Gaithersburg, Maryland, and white colonies were isolated and screened for proper insert size to yield the plasmid pCDW. To produce a suitable plasmid based expression vector for the CD4 gene, the plasmid SV2neo, obtained from American Type Culture Collection, Rockville, Maryland, was digested with Hind III plus Hpa I. A synthetic polylinker sequence from the pUC-13 vector from Pharamicia, Piscataway, New Jersey, was inserted via T4 DNA ligase in place of the Neo^R gene of PsV2neo. This ligation was transformed into DH5 bacteria from Bethesda Research Labs and colonies screened for the presence of restriction enzyme sites unique to the polylinker to yield the vector pSVPL. The pSCPL expression vector was further modified by the insertion of an Xho I linker using conditions and reagents suggested and supplied by New England Biolabs, into the Pvu II site on the 5' side of the SV40 early region promoter to produce pSVPLX.

The pCDW and pSVPLX plasmids were digested with enzymes Hind III plus Xba I from New England Biolabs and their DNAs isolated using the Geneclean product following agarose gel electrophoresis. Ligation of the CD4

fragment into the pSVPLX vector was performed and colonies were screened to yield pSVCDW in which the SV40 virus early region promoter is used to drive the expression of the complete CD4 gene product. The next step was to produce a form of the CD4 gene such that it would be exported from the cell as an extracellular product.

C. The production of a soluble form of CD4 was accomplished by the use of a specially designed oligonucleotide adaptor to produce a mutant form of the CD4 gene. This adaptor has the unique property that when inserted into the Nhe I site of the CD4 gene it produces the precise premature termination of the CD4 protein amino acid sequence while regenerating the Nhe I site and creating a new Hpa I site. This oligonucleotide adaptor, synthesized by Midland Certified Reagent Co., was produced by annealing two phosphorylated oligonucleotides: (1) 5'CTAGCITGAGTGAGIT 3' and (2) AACTCACTCAAG. This product was then ligated into the site of pSVCDW. The ligation reaction was then cleaved with Hpa I and then Xho I linkers were added. The linker reaction was terminated by heating at 65°C for 15 minutes and then subjected to digestion with Xho I restriction endonuclease from New England Biolabs. This reaction was then subjected to agarose gel electrophoresis and the fragment containing the SV40-CD_4 adaptor isolated using the Geneclean product. The retroviral vector N2 was prepared to accept the SV40-CD_4-adaptor fragment by digestion with Xho I and treatment with calf intestinal phosphatase from Boehringer Mannheim, Indianapolis, Indiana.

The ligation of a CD4 expression cassette was performed with an insert to vector ratio of 5:1 and then transformed in DH5 competent bacteria from Bethesda Research Labs. Constructs were analyzed by restriction endonuclease digestion to screen for orientation and then grow up in large scale. The construct where the SV40 virus promoter is in the same orientation as the viral LTR promoters is known as SSC while the construction in the reverse orientation is called SCSX.

The SSC vector is packaged into PA 317 cell line as described by Miller, et al., <u>supra</u>, to provide PA 317 cells capable of producing soluble CD4 protein. The SSC vector packaged PA 317 cells were used to transduce rabbit endothelial cells as described above. The transduced endothelial cells expressed soluble CD4.

D. Collagen sponges containing adsorbed HBGF-1 of the type previously described were surgically implanted in the abdominal cavity of a rat near the liver. Sponges were surgically removed seven to ten days post-implantation and digested 30 to 60 minutes at 27°C with a solution of collagenase in phosphate buffered saline in a concentration of 1mg/ml using a tissue culture incubator at 5 percent in CO_2. Released cells were collected by centrifugation for 10 minutes at 1000 RPM at 20°C. The cells were washed once with phosphate buffered saline (PBS) and pelleted by centrifugation. Cells were resuspended with two volumes of 30 ml of media containing: M199 media (Gibco); ECGF (crude brain extract) 7.2mg; Heparin (Upjohn) 750 units;

and 20 percent conditioned cellular media collected as supernatant from confluent dishes after 48 hours of either bovine aortic or human umbilical vein endothelial cells. The other media contained: 10 percent fetal calf serum (Hyclone); 3000 units Penicillan G (Biofluids); and 3000 units streptomycin sulfate (Biofluids) and the cells were plated for 16 hours on 100 mm tissue culture disk coated with fibronectin (human) using $1ug/cm^2$. Plated cells were washed with PBS three times and fed 15ml of previously mentioned media. Media was changed every 2 days for the duration of the procedures.

Selected rat endothelial cells were transduced with N-7, SAX, G2N and SSC vectors by the following procedures:

1. 2×10^6 microendothelial cells (monolayer 80 percent confluent)

2. 2×10^6 cfu/ml viral supernatant

3. Polybrene (8ug/ml)

- Combine 1, 2, 3 in 5 ml total volume for 2-3 hours at 37°C (5 percent CO_2).

- Add 20ml of tissue culture media for 16 hours, at 37°C (5 percent CO_2).

- Aspirate off media (virus containing), add fresh culture media.

- After 48-96 hours, add G418 (800ug/ml) and culture media.

- Select for one to two weeks changing media every two days.

The following are procedures for seeding a sponge with the transduced endothelial cells described above.

A. The endothelial cells are seeded directly onto a HBGF-1 adsorbed, collagen coated PTFE fiber sponge, and the sponge is implanted back into the same animal used as the source of endothelial cells. The site of implantation can be subcutaneous, intraperitoneal, or at or near the site of the organ that normally produces the new product encoded by the gene transduced into the endothelial cells. The sponge implant generates its own vascularization within 5 to 10 days, as described in earlier examples. The engineered endothelial cells are maintained on the implant such that the new gene product is delivered directly into the circulation after secretion from the cell. The production of the gene product is monitored either by direct measurement of its serum levels, by the biochemical or physiological effect of the agent, or both.

B. An HBGF-1 absorbed, collagen coated PTFE fiber sponge is preimplanted at the desired site, as described above, and at the time determined to be optional for that implant site for establishment of neovascularization. The transformed cells are injected directly into the

already-vascularized fiber sponge. The advantage of this method is that the engineered cells are more rapidly and effectively established in the implant or migrate back into the parent organ (e.g., liver). The product begins to enter the circulation much sooner than with method A above. Production of the new gene product is measured as described in method A. This procedure can be applied to a number of different cell types capable of being sampled, genetically engineered in vivo, and reinserted via the fiber sponge implant. Such cells include fibroblasts, hepatocytes, smooth muscle cells, bone marrow cells and others. The products delivered to the circulation can be any peptide or protein whose gene can be inserted into a cell and whose product is desired to be delivered.

EXAMPLE 7

Gortex shunt tubes were surgically implanted into the peritoneum of rats, in such a way as to form a loop, with each end contacting the aorta. The tubes contained either a Gelfoam (Collagen I) sponge impregnated with HBGF-1 (1 ng/ml) or a bundle of "angel hair" Gortex fibers, coated with Collagen I and impregnated with HBGF-1 (1 ng/ml). The tubes were left in the animals for one month, then surgically extracted, grossly examined for blood vessel formation, and the sponge prepared for histological examination. As shown in Figure 14A, the

tube that had contained the Gelfoam sponge contained no new blood vessels, and the sponge had completely dissolved. In contrast, the angel-hair Gortex fiber bundles became significantly vascularized (Figure 14B), with higher magnification showing the capillary structures (Figures 14C, D).

This experiment provides an example of directing neovascularization to a particular site, with a two component device. The first component, a tube or pouch, can provide a receptacle in which implanted cells, genetically engineered or normal, can be seeded. It is possible that such a site may be immunologically privileged, and allow cells from another individual, or even another species, to survive and produce a desired product.

WHAT IS CLAIMED IS

1. A neovascularization device comprising

a biocompatible support; and

a biological response modifier for inducing neovascularization, said biological response modifier being adsorbed to said biochemical support.

2. The neovascularation device of claim 1 wherein said biocompatible support is an absorbable support.

3. The neovascularization device of claim 2 further comprising:

a non-absorbable support.

4. The neovascularization device of claim 1 wherein said biocompatible support is a non-absorbable support.

5. The neovascularization device of claim 2 wherein said absorbable support is a member selected from the group consisting of collagen, laminin, fibronectins, gelatin, glycosaminoglycan, glycoproteins, proteoglycans and mixtures thereof.

6. The neovascularization device of claim 1 wherein said biological response modifier is a member selected from the group consisting of a hormone, a hormone prototype, a hydrolase, and mixtures thereof.

7. The neovascularization device of claim 6 wherein said hormone is an angiogenic and neurotrophic growth factor being a member selected from the group consisting of HBGF-I, HBGF-II, an HBGF-I prototype, an HBGF-II prototype, and mixtures thereof.

8. The neovascularization device of claim 6 wherein said hydrolase is heparinase, collagenase, plasmin, a plasminogen activator, thrombin, heparatinase, and mixtures thereof.

9. The neovascularization device of claim 1 wherein said biological response modifier is an angiogenic growth factor, said angiogenic growth factor being in a concentration of about 1 to about 10 nanograms per mm^3 of said support.

10. The neovascularization device of claim 3 wherein said non-absorbable support is a member selected from the group consisting of nylon, rayon, dacron, polypropylene, polyethylene, PTFE, collagen I, collagen IV, kerratin, and glycolipid.

11. The neovascularization device of claim 4 wherein said non-absorbable support is a member selected from the group consisting of nylon, rayon, dacron, polypropylene, polyethylene, PTFE, collagen I, collagen IV, kerratin, and glycolipid.

12. The neovascularization device of claim 2 wherein said absorbable support is gelatin.

13. A neovascularization device comprising:

an absorbable support;

a non-absorbable support, said absorbable support being adsorbed to said non-absorbable support; and

a biological response modifier in sufficient concentration for inducing <u>in vivo</u> site directed neovascularization, said biological response modifier being adsorbed to said absorbable support.

14. The neovascularization device of claim 13 wherein said absorbable support is a member selected from the group consisting of collagen, laminin, fibronectins, gelatin, glycosaminoglycan, glycoproteins, proteoglycans and mixtures thereof.

15. The neovascularization device of claim 13 wherein said biological response modifier is a member selected from the group consisting of a hormone, a hormone prototype, a hydrolase, and mixtures thereof.

16. The neovascularization device of claim 15 wherein said hormone is an angiogenic and neurotrophic growth factor being a member selected from the group consisting of HBGF-I, HBGF-II, an HBGF-I prototype, an HBGF-II prototype, and mixtures thereof.

17. The neovascularization device of claim 15 wherein said hydrolase is heparinase, collagenase, plasmin, a plasminogen activator, thrombin, heparatinase, and mixtures thereof.

18. The neovascularization device of claim 13 wherein said biological response modifier is an angiogenic growth factor, said angiogenic growth factor being in a concentration of about 1 to about 10 nanograms per mm^3 of said per mm^3 of both said absorbable support and non-absorbable support.

19. The neovascularization device of claim 13 wherein said non-absorbable support is a member selected from the group consisting of nylon, rayon, dacron, polypropylene, polyethylene, PTFE, collagen I, collagen IV, kerratin, and glycolipid.

20. A neovascularization device comprising:

a biocompatible support; and

a biological response modifier for inducing in vivo site directed neovascularization, said biological responses modifier being (i) in a concentration of about 1 to about 10 nangrams per mm^3 of said biocompatible support and (ii) a member of the group consisting of a hormone, a hormone prototype, a hydrolase, and mixtures thereof.

21. The neovascularation device of claim 20 wherein said biocompatible support is an absorbable support.

22. The neovascularization device of claim 21 further comprising:

a non-absorbable support.

23. The neovascularization device of claim 20 wherein said biocompatible support is a non-absorbable support.

24. The neovascularization device of claim 21 wherein said absorbable support is a member selected from the group consisting of collagen, laminin, fibronectins, gelatin, glycosaminoglycan, glycoproteins, proteoglycans and mixtures thereof.

25. The neovascularization device of claim 20 wherein said hormone is an angiogenic and neurotrophic growth factor being a member selected from the group consisting of HBGF-I, HBGF-II, an HBGF-I prototype, an HBGF-II prototype, and mixtures thereof.

26. The neovascularization device of claim 20 wherein said hydrolase is heparinase, collagenase, plasmin, a plasminogen activator, thrombin, heparatinase, and mixtures thereof.

27. The neovascularization device of claim 22 wherein said support is a member selected from the group consisting of nylon, rayon, dacron, polypropylene, polyethylene, PTFE, collagen I, collagen IV, kerratin, and glycolipid.

28. The neovascularization device of claim 23 wherein said non-absorbable support is a member selected from the group consisting of nylon, rayon, dacron, polypropylene, polyethylene, PTFE, collagen I, collagen IV, kerratin, and glycolipid.

29. A process for producing neovascularization comprising:

adsorbing a biological response modifier for inducing neovascularization onto a biocompatible support;

contacting a therapeutically effective amount of said adsorbed biological response modifier to at least one selected tissue in an organism; and

directing *in vivo* growth of neovascular cells at said contacted, selected tissue for a sufficient time to obtain a vascular structure.

30. The process for producing neovascularization of claim 29 wherein said neovascular cells contain a genetic insert.

31. The process for producing neovascularization of claim 30 wherein said genetic insert enables said neovascular cells to secrete a biological product.

32. The process for producing neovascularization of claim 31 wherein said biological product is a biological response modifier.

33. The process for producing neovascularization of claim 32 wherein said biological response modifier is a member selected from the group consisting of a hormone, a hormone precursor, and a hydrolase.

34. The process for producing neovascularization of claim 29 further comprising:

seeding said vascular structure with non-vascular cells.

35. The process for producing neovascularization of claim 34 wherein said seeded cells secrete a desired biological product.

36. The process for producing neovascularization of claim 34 wherein said seeded cells perform a desired metabolic function.

37. The process for producing neovascularation of claim 29 wherein said biocompatible support is an absorbable support.

38. The neovascularization device of claim 37 further comprising:

a non-absorbable support.

39. The neovascularization device of claim 29 wherein said biocompatible support is a non-absorbable support.

40. The neovascularization device of claim 37 wherein said absorbable support is a member selected from the group consisting of collagen, laminin, fibronectins, gelatin, glycosaminoglycan, glycoproteins, proteoglycans and mixtures thereof.

41. The neovascularization device of claim 29 wherein said biological response modifier is a member selected from the group consisting of a hormone, a hormone prototype, a hydrolase, and mixtures thereof.

42. The neovascularization device of claim 41 wherein said hormone is an angiogenic and neurotrophic growth factor being a member selected from the group consisting of HBGF-I, HBGF-II, an HBGF-I prototype, an HBGF-II prototype, and mixtures thereof.

43. The neovascularization device of claim 41 wherein said hydrolase is heparinase, collagenase, plasmin, a plasminogen activator, thrombin, heparatinase, and mixtures thereof.

44. The neovascularization device of claim 29 wherein said biological response modifier is an angiogenic growth factor, said angiogenic growth factor being in a concentration of about 1 to about 10 nanograms per mm^3 of said support.

45. The neovascularization device of claim 38 wherein said non-absorbable support is a member selected from the group consisting of nylon, rayon, dacron, polypropylene, polyethylene, PTFE, collagen I, collagen IV, kerratin, and glycolipid.

46. The neovascularization device of claim 39 wherein said non-absorbable support is a member selected from the group consisting of nylon, rayon, dacron, polypropylene, polyethylene, PTFE, collagen I, collagen IV, kerratin, and glycolipid.

47. A product for promoting neovascularization, comprising:

a support including an extracellular matrix protein and a biological response modifier.

48. The product of claim 47 wherein the support includes cells capable of expressing a metabolite whereby the product is capable of inducing organoid neovascularization.

49. The product of claim 48 wherein the cells are genetically engineered to express a heterologous protein.

50. The product of claim 49 wherein the support is a non-absorbable support.

51. The product of claim 50 wherein the biological response modifier is absorbed to the extracellular matrix protein included in the non-absorbable support.

52. The product of claim 51 wherein said biological response modifier is a member selected from the group consisting of a hormone, a hormone prototype, a hydrolase, and mixtures thereof.

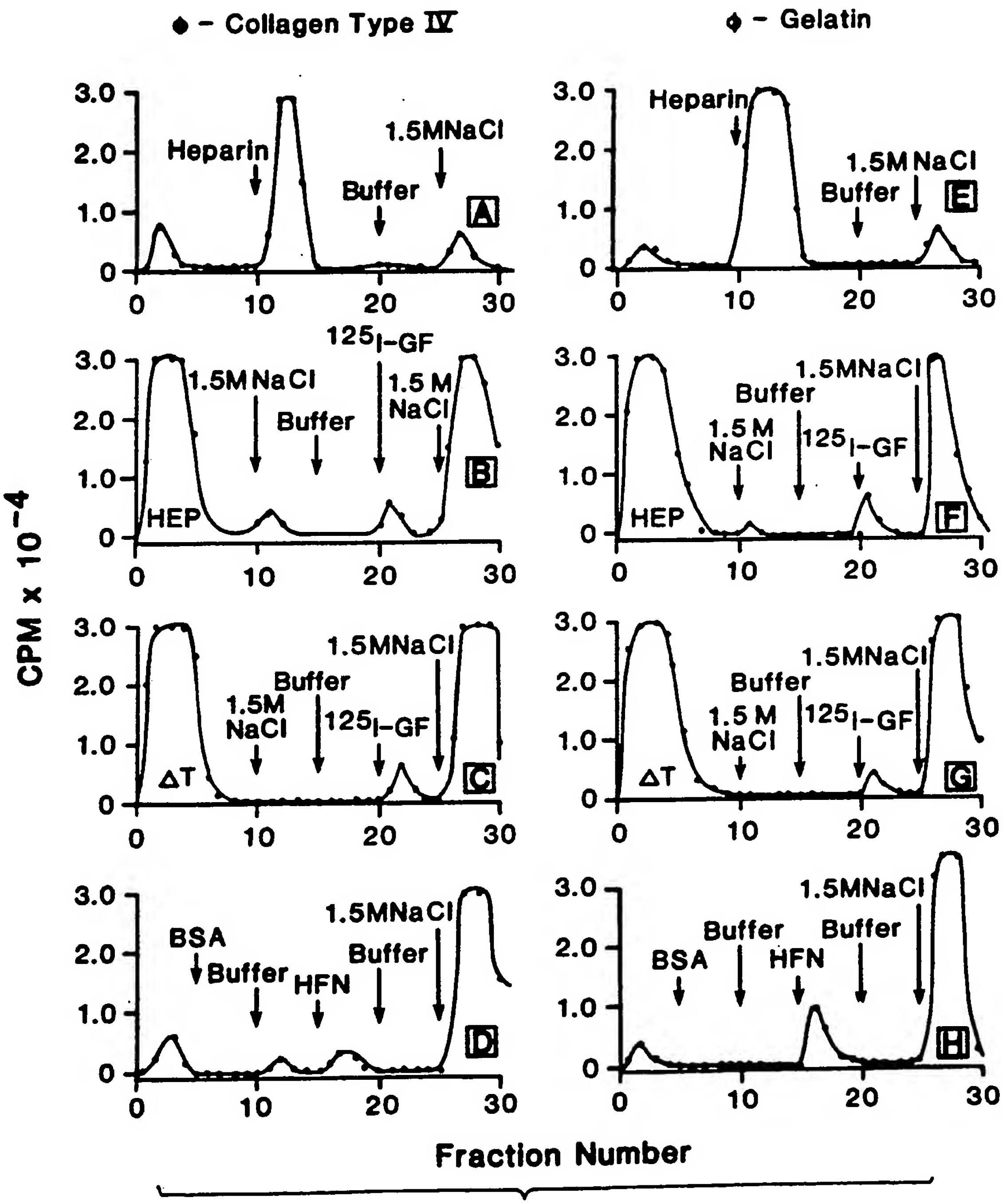
53. The product of claim 52 wherein the biological response modifier is at least one member selected from the group consisting of heparinase, collagenase, plasmin, a plasminogen activator, thrombin, and heparatinase.

54. The product of claim 52 wherein the biological response modifier is at least one member selected from the group consisting of HBGF-I, HBGF-II, and HBGF-I prototype, and an HBGF-II prototype.

55. The product of claim 51 wherein said biological response modifier is an angiogenic growth factor, said angiogenic growth factor being in a concentration of about 1 to about 10 nanograms per mm^3 of said support.

56. The product of claim 51 wherein said non-adsorbable support is a member selected from the group consisting of nylon, rayon, dacron, polypropylene, polyethylene, PTFE, and cross-linked collagen IV.

57. The product of claim 51 wherein the extracellular matrix protein is at least one member selected from the group consisting of collagen, laminin, fibronectins, gelatin, glycosaminoglycan, glycoproteins, and proteoglycans.



● - Collagen Type IV
◆ - Gelatin
CPM x 10^-4
Fraction Number
Heparin
Buffer
1.5MNaCl
125I-GF
HEP
ΔT
BSA
HFN
A
B
C
D
E
F
G
H

FIG. 1

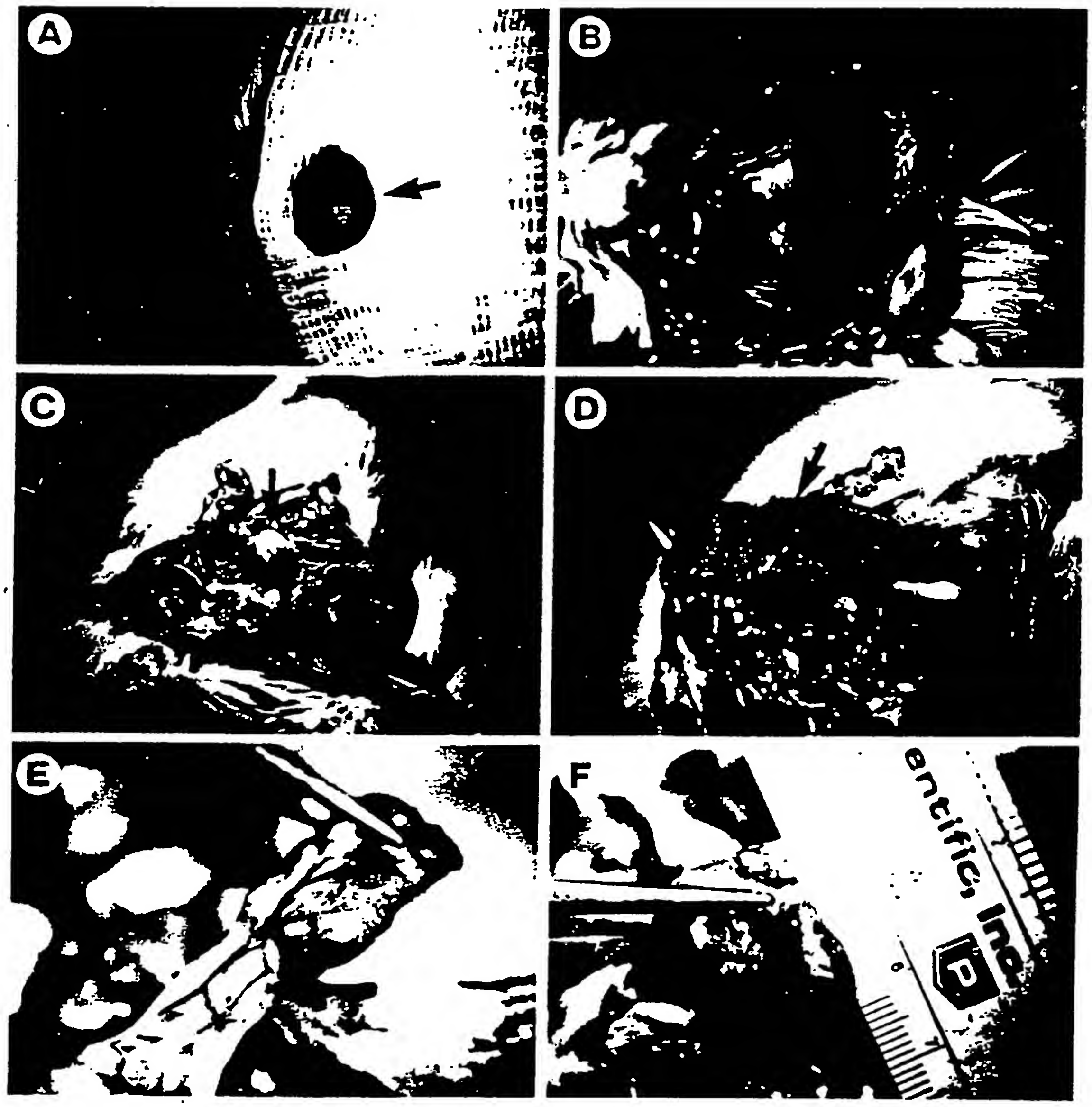


FIG. 2

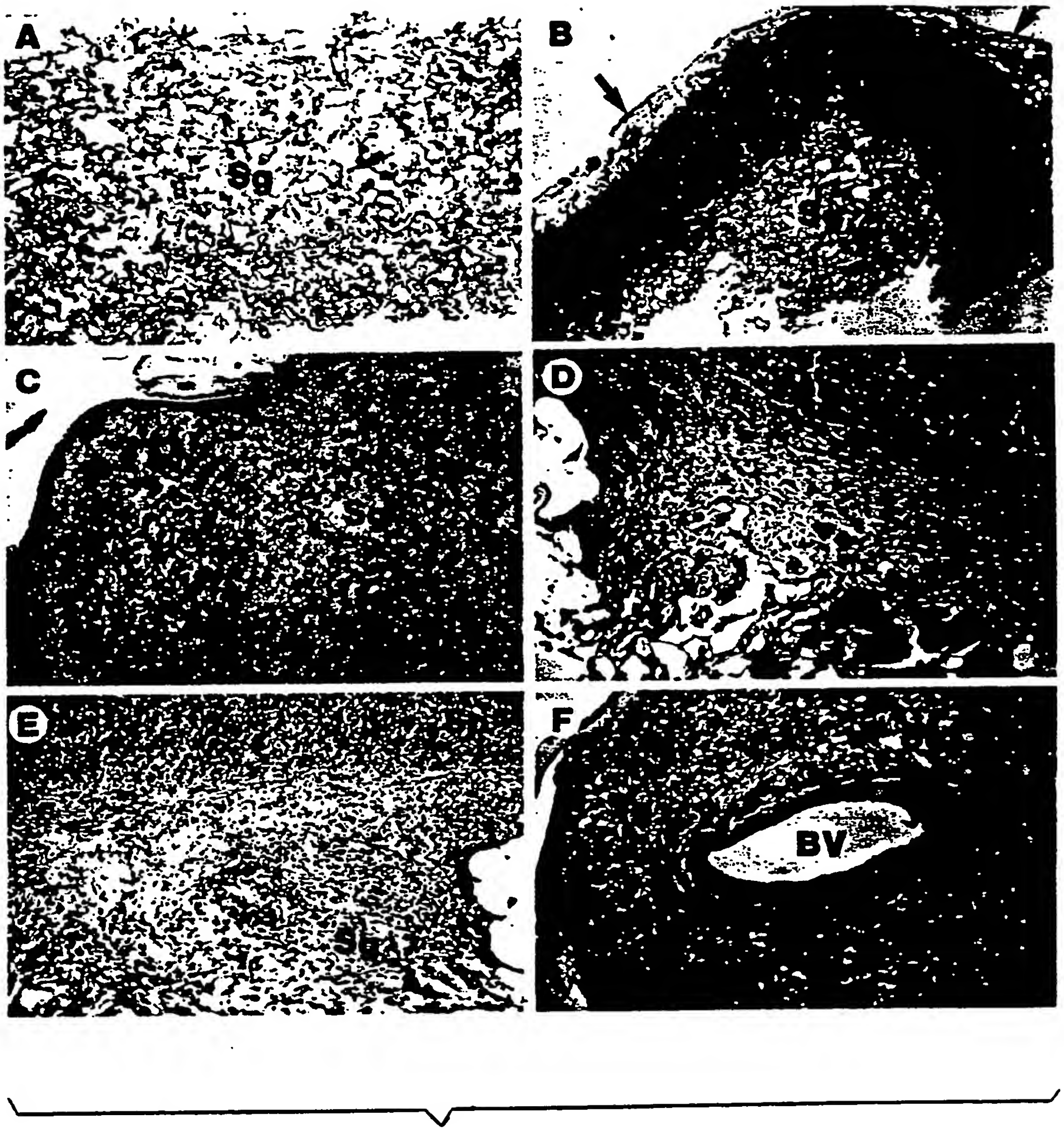


FIG. 3

FIG. 4

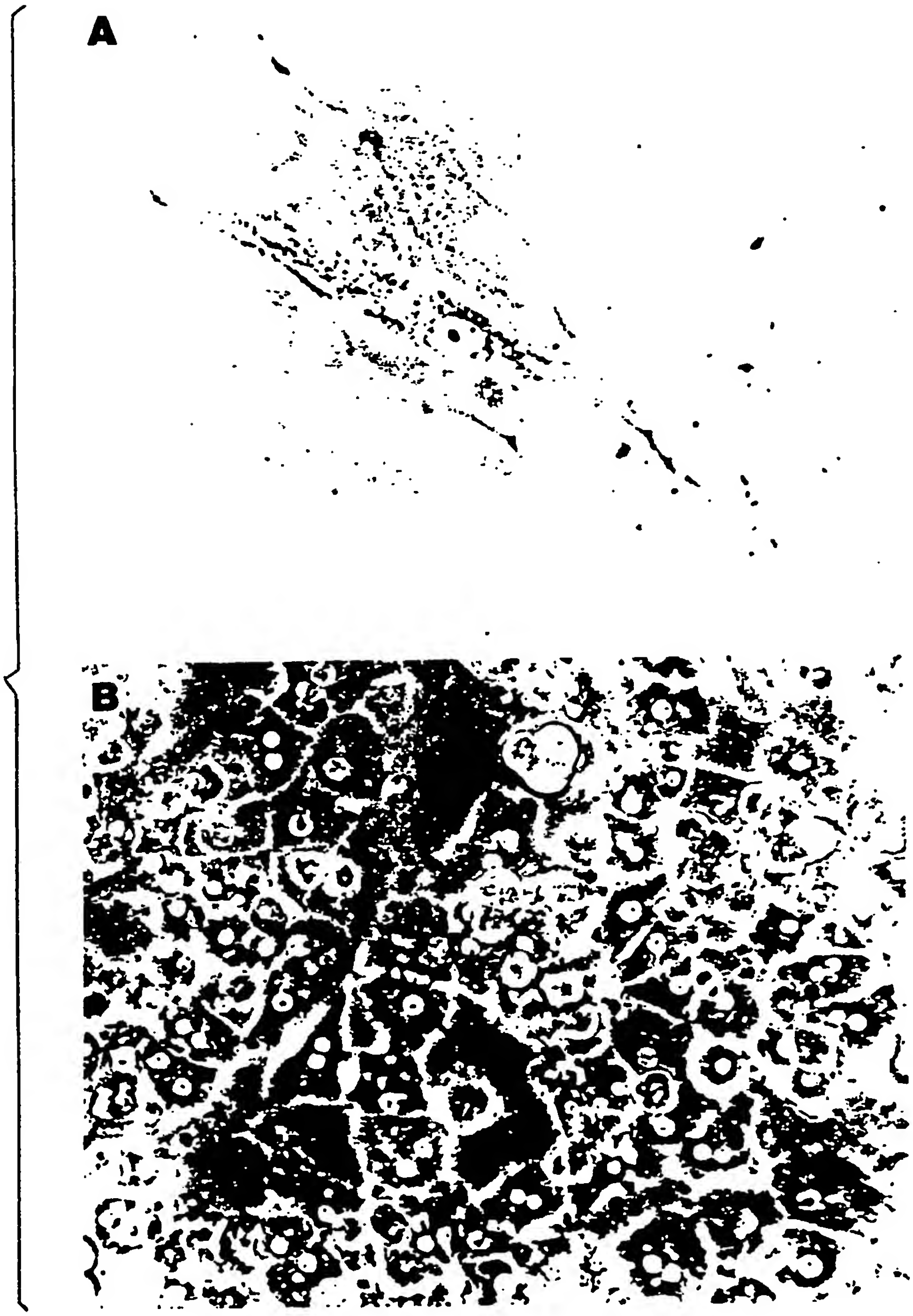


FIG. 5

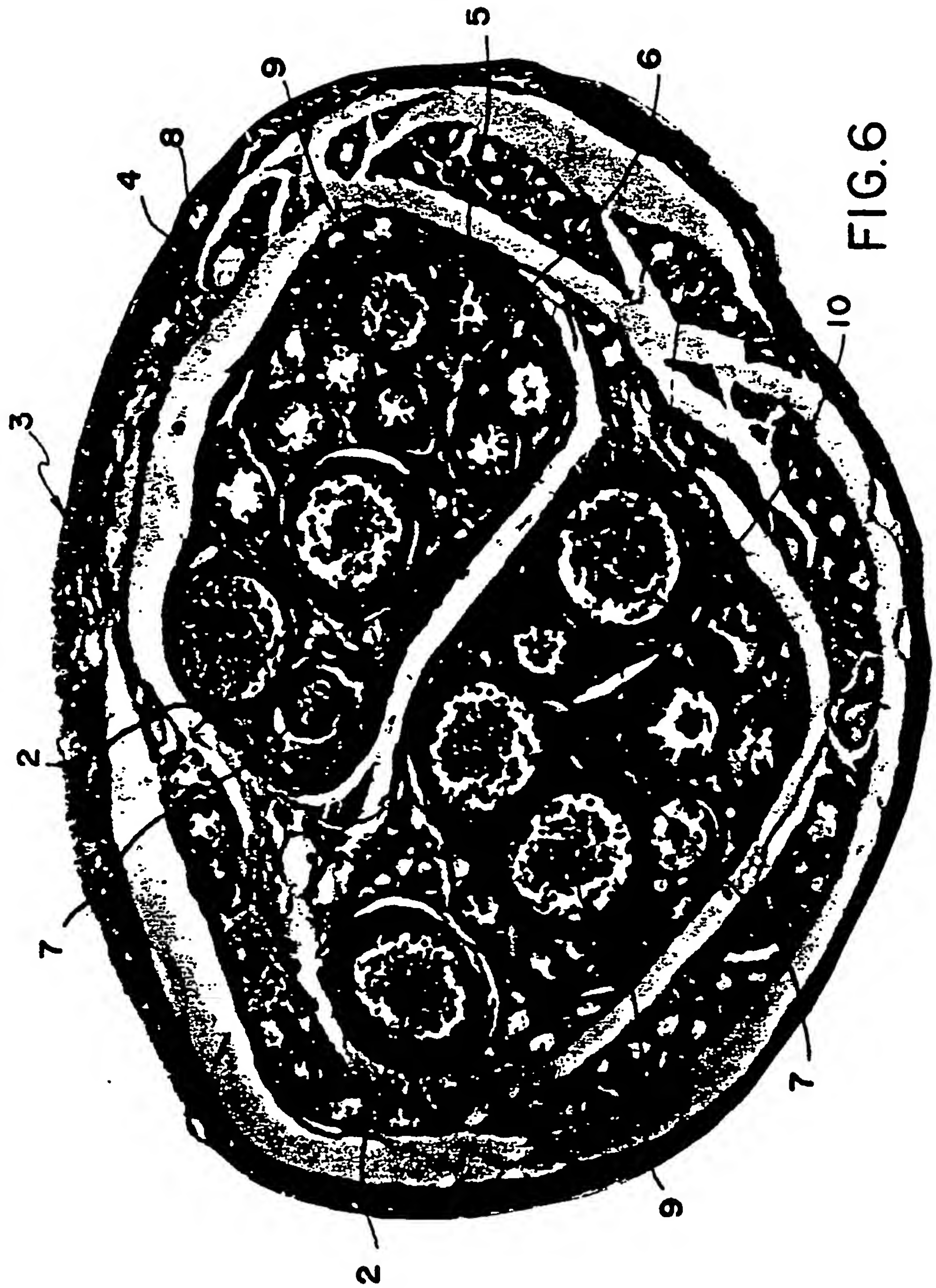


FIG.6

FIG. 7

FIG. 8

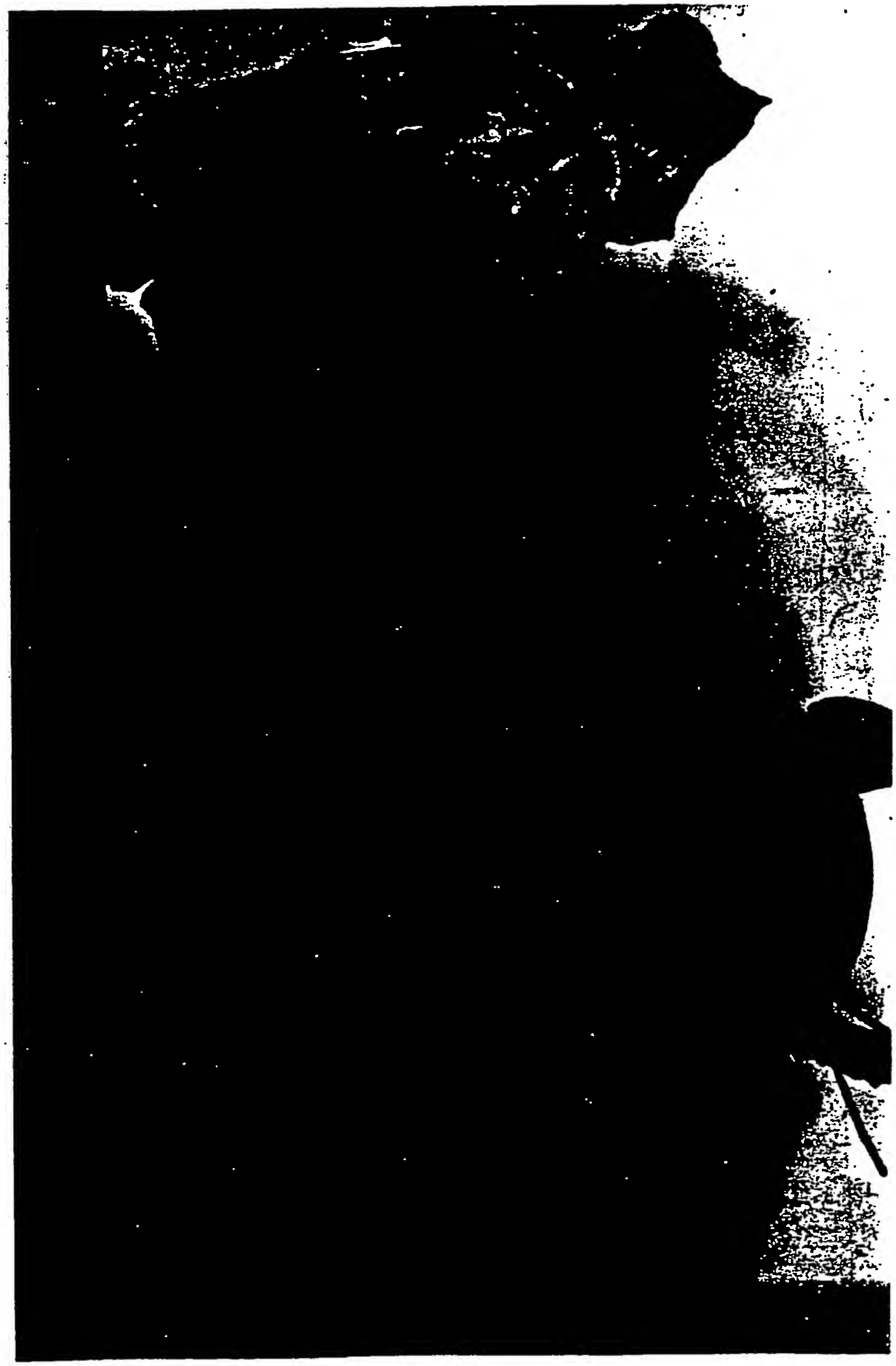


FIG. 9

FIG. 10

FIG. 11

FIG. 12

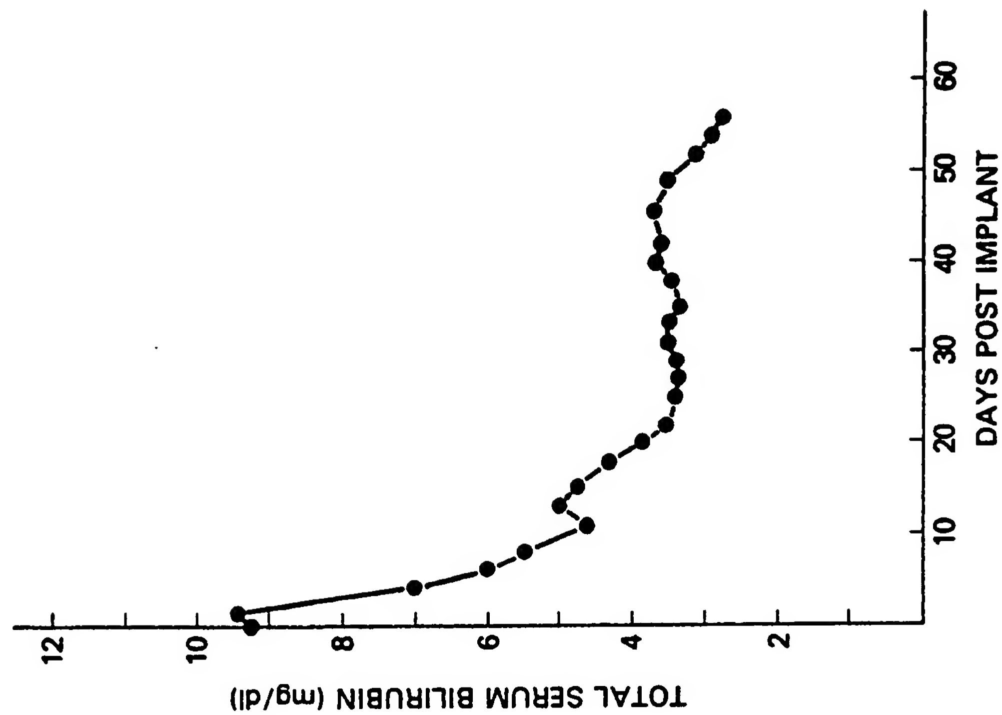


FIG. 13B

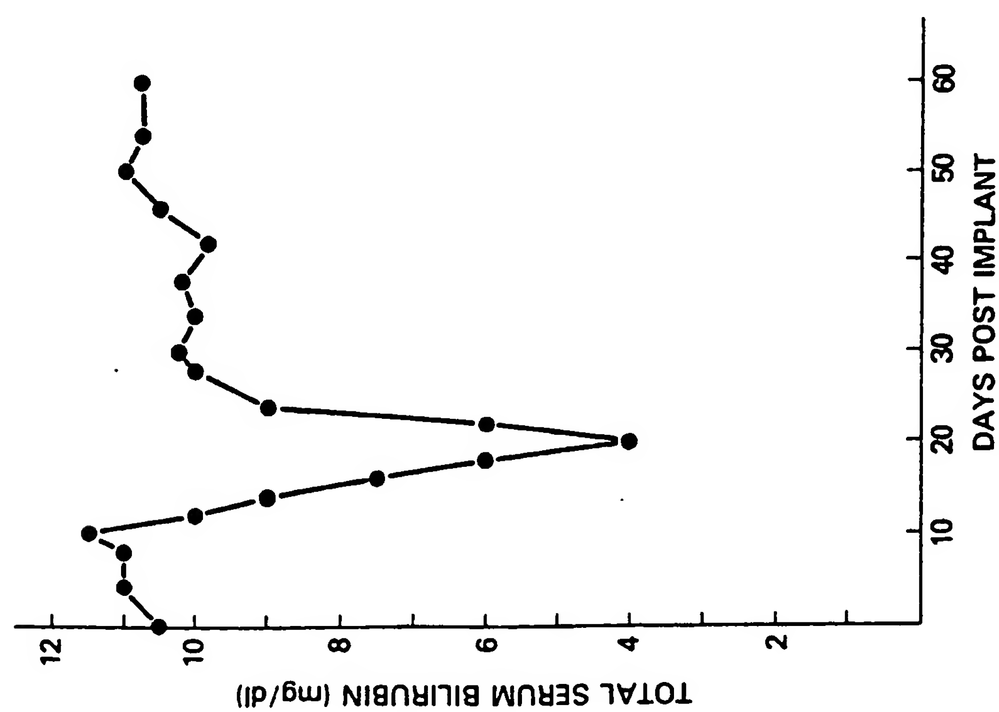


FIG. 13A

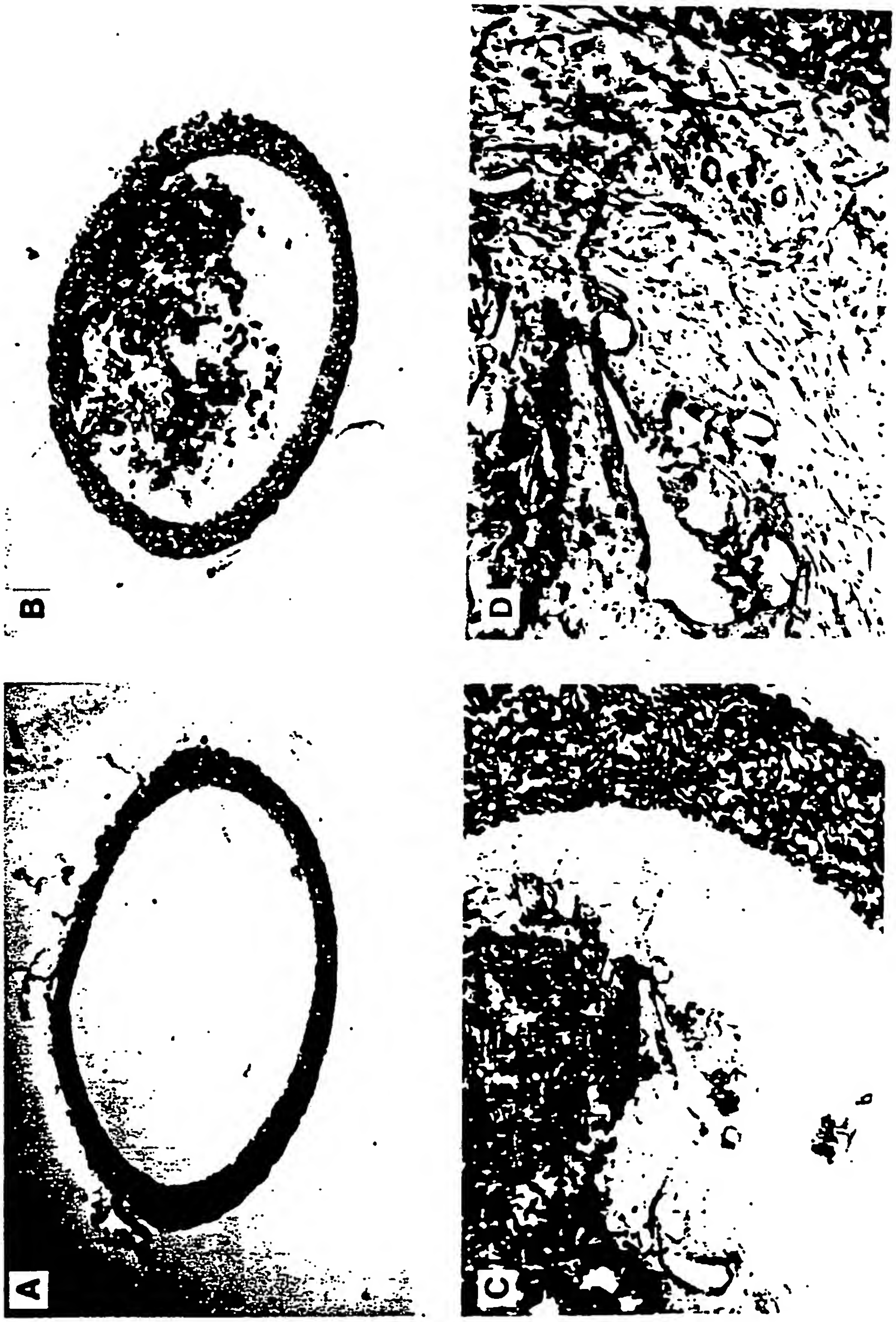


FIG. 14

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/00742

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A61K 37/02,37/24,37/54;C12N 5/00,11/02,11/08

U.S./Cl.: 514/2,8;424/94.6,94.61,94.63,94.64;435/177,180,240.23

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/94.6,94.61,94.63,94.64;435/177,180,240.23 514/2,8,21,774,801

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

DATABASES: CHEMICAL ABSTRACTS SERVICES ONLINE (FILE CA, 1967-1989; FILE BIOSIS, 1969-1989). See Attachment for Search terms.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,699,141 (Lamberton et al) 13 October 1987, See Entire Document	13-16,18, 19,22,27, 30-38,40, 42,45, 47-52, 54-57
X	US, A, 4,699,141 (Lamberton et al) 13 October 1987, See Entire Document	1,4,29, 34,35,36, 39,47,48
Y	British Journal of Experimental Pathology Volume 68, Issued 1987, Andrade et al, "Quantitative in Vivo Studies on angiogenesis in a rat sponge model", pages 755-766, Entire Document.	1,4,6, 9-11,20, 23,28,29, 39,41,44, 46
Y	Proceedings of the National Academy of Sciences, USA, Volume 82, Issued November 1985, Buckley et al, "Sustained release of epidermal growth factor accelerates wound repair," pages 7340-7344, Entire Document.	1,4,6,9, 11,20,23, 28,29,39, 41,44,45, 46

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09 May 1989	05 JUL 1989
International Searching Authority ISA/US	Signature of Authorized Officer Gail F. Knox Gail F. Knox

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X Y	Biochemical and Biophysical Research Communications Volume 147, Issued 15 September 1987 Hayek et al, "An In Vivo model for study of the angiogenic effects of basic fibroblast growth factor", pages 876-880, Entire document.	1-7, 12-16, 29, 37-42 9-11, 18-25, 27-28, 44-46

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers........ , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers___________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	WO, A 8701728 (Biotechnology Research Partners, LTD) 26 March 1987, pages 1-64, See pages 1-3, 12-16.	1,4,6, 7,9,11, 20,23, 25,28, 29,39, 44,46
X,P	Journal of Cell Biology, Volume 107 (6 part 3), Issued 09 December 1988, Maciag et al, "Heparin-Binding Growth Factor-I (HBGF-I) Bound to Gelatin Induces Site-Specfic Neo-vessel Formation In Vivo", Abstract No. 2697, page 479a.	1-57
Y	Surgical Science Series, Volume 2, Issued 1984, Jackson et al," Effect of Angiogenic Factors on the Vascularization of IVALON Sponge Implants", pages 190-201, See entire document.	1,4,6, 7,9, 11,20, 23,25, 28,29 39,44, 46
Y	Biochemical and Biophysical Research Communications, Volume 142, Issued 30 January 1987, Baird et al, "Fibroblast Growth Factors are Present in the Extracellular Matrix Produced by Endothebal Cells <u>In Vitro</u>. Implications for a Role of heporinase-like Enzymes in the Neovascular Response, pages 428-435, especially page 433.	8,17, 26,43, 53
Y	Cancer Research, Volume 43, Issued June 1983, Vlodavsky et al, "Lymphona Cell-Mediated Degradation of sulfated Proteoglycans in the Subendothelial Extracellular Matrix: Relationship to Tumor Cell Metastiasis", pages 2704-2711, especially page 2710.	8,17, 26,43, 53
Y	Chemical Abstracts, Volume 109, Issued 10 October 1988, Rifkin, "Endothelial Cell proteases and Cellular invasion", Abstract No. 123103	8,17, 26,43 53

Attachment to Form PCT/ISA/210
Part II. FIELDS SEARCHED SEARCH TERMS:

Angiogenesis
Neovascularization
IVALON
Neovessel
formation
sponge
support
biocompatible
transplant
heparinase
heparitinase
collagenase
plasminogen
activator
plasmin
hydrolase